Title: **Designer T-cells and T-cell receptors for customised cancer immunotherapies**

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Abstract

Cancer immunotherapy, focused on harnessing and empowering the immune system against tumours, has transformed modern oncology. One of the most promising avenues in development involves using genetically engineered T-cells to target cancer antigens *via* specific T-cell receptors (TCRs). TCRs have a naturally low affinity towards cancer-associated antigens, and therefore show scope for improvement. Here we describe approaches to procure TCRs with enhanced affinity and specificity towards cancer, using protein engineering or selection of natural TCRs from unadulterated repertoires. In particular, we discuss novel methods facilitating the targeting of tumour-specific mutations. Finally, we provide a prospective outlook on the potential development of novel, off-the-shelf immunotherapies by leveraging recent advances in genome editing.

Abstract: 111 words (100-120)

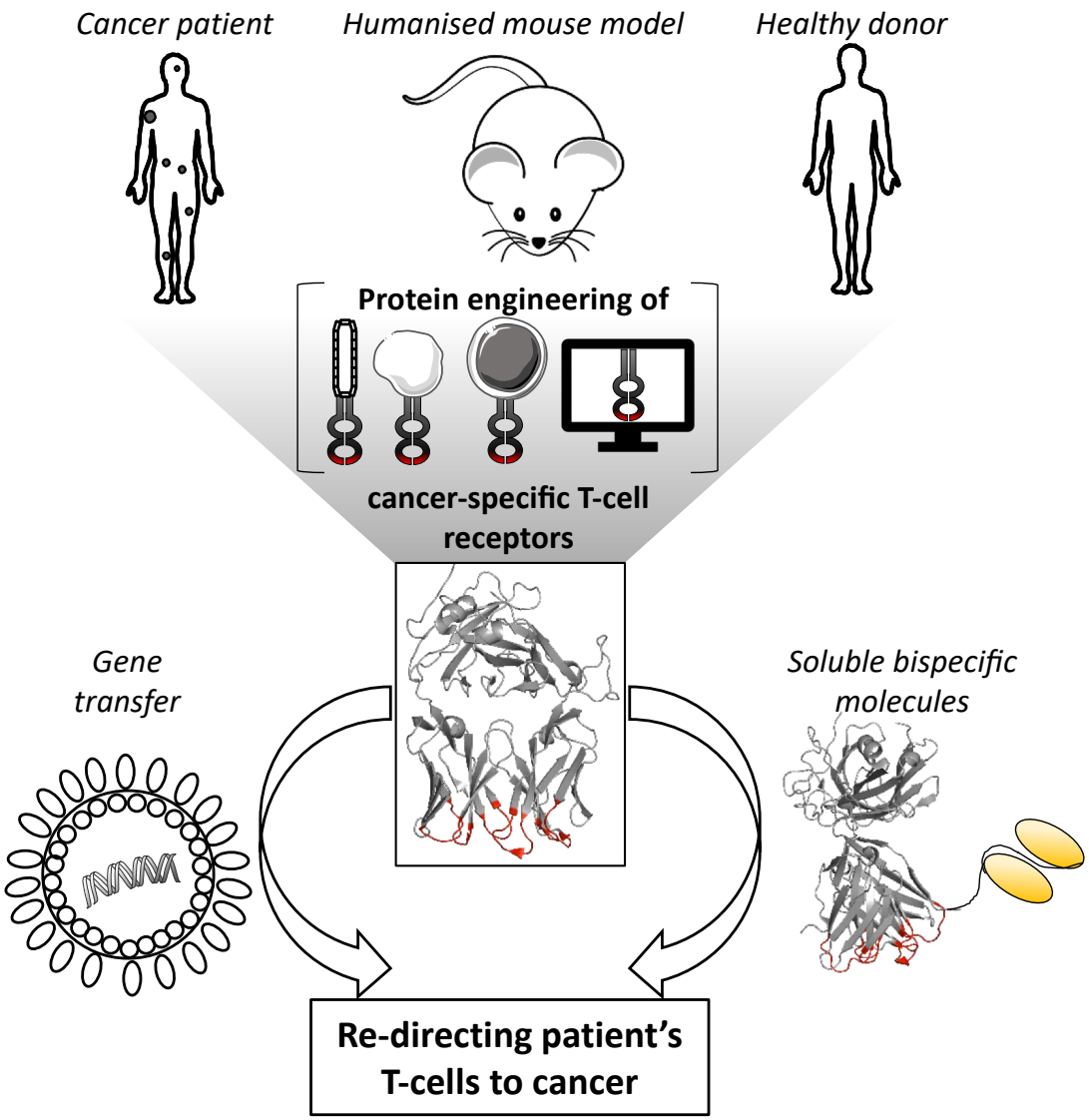
Main text: 1866 ($<$2,000)

Objects: (up to 4) – 1 table, 3 figures (+graphical abstract)

References: 53 ($\sim$50) – and around 50% are from 2016&2017

Highlights (3-5, 85 characters each):

- T-cell receptors (TCRs) can be used to re-direct patients' immune system to cancer
- Directed evolution can enhance TCR affinity towards cancer antigens
- Affinity-enhanced TCRs can be used in gene transfer or as soluble molecules
- TCR targeting of somatic mutations offers personalised, safe and specific therapy
- Modern genome engineering offers promise of new improved immunotherapies



Legut and Sewell, graphical abstract

Introduction

Conventional cytotoxic $CD8^+$ T-cells recognise short peptide antigens, derived from degradation of intracellular proteins and presented by molecular cradles called the major histocompatibility complex (MHC, also known as human leukocyte antigen, HLA) class I at the cell surface (reviewed in [1]). This recognition allows T-cells to scan the intracellular proteome for anomalies and destroy cells expressing foreign, pathogen-derived proteins. Recognition of the highly variegated MHC-associated peptide cargo is possible thanks to generation of diverse αβ T-cell receptors (TCRs) by somatic gene rearrangement process that has potential to theoretically generate $\sim 10^{18}$ different receptors in human [2]. Immature T-cells are then selected in the thymus so that only those with TCRs that weakly engage self MHC molecules are allowed to enter the periphery. Cells that bear inept TCRs "die by neglect" while those that recognise self peptides strongly, and have capacity to induce autoimmunity, are culled [3] Thus, the TCRs of T-cells that populate the periphery should not be self-reactive but have potential to bind foreign peptides with relatively high affinity. This central tolerance mechanism restricts the TCR pool with anticancer specificity to predominantly weak affinity receptors [4] because the majority of cancer antigens, with a notable exception of neoantigens stemming from mutated proteins, are derived from over- or aberrantly expressed self-proteins [5] (**Figure 1**). The challenge that this presents to anti-tumour T-cells is further amplified because successful tumours exploit a variety of systems that are designed to protect against excessive T-cell activity in the periphery [6]. Neutralisation of peripheral tolerance mechanisms via the application of immune checkpoint inhibitors has shown great promise in cancer treatment but is associated with concomitant autoimmune toxicities [7]. More targeted approaches in current development aim to adoptively transfer cancer-reactive T-cells. Adoptive T-cell therapy originally involved transfer of expanded tumour infiltrating lymphocytes [8] but has been extended to include autologous T-cells engineered *ex vivo* to express well-defined, cancer-specific receptors. The genetic redirection of T-cells to cancer can be achieved by using antibody-like chimeric antigen receptors (CARs) targeting surface-expressed proteins (which became the first gene therapy approved by the FDA [9]), or via conventional TCRs which can scan the intracellular proteome presented as peptide-MHC complexes. Here we discuss the recent developments in procurement of the optimal TCRs for cancer immunotherapy and cutting-edge technologies that enable high throughput, in-depth assessment of potentially therapeutic TCRs.

The critical importance of TCR affinity

The first-in-human attempt to re-direct patient T-cells to cancer was conducted in 2006 by Rosenberg and colleagues and used the DMF4 TCR that targets a peptide from a melanocyte differentiation antigen highly expressed in melanoma [10]. While the results of that clinical trial were encouraging in terms of safety and feasibility, only a relatively small fraction of patients experienced an objective response (**Table 1**). The clinical response was improved in the follow up trial which made use of another *natural* TCR targeting the same epitope as DMF4 but selected by screening of hundreds of T-cell clones for TCRs exhibiting substantially higher sensitivity [11,12]. These two trials therefore demonstrated the importance of T-cell sensitivity to antigen, a property known to be critically dependent on TCR affinity/half-life [13]. As natural anti-cancer TCRs are of low affinity, several strategies have been devised to generate TCRs with optimal affinity as described below.

Directed evolution can create super-high affinity TCRs

The first robust method for affinity maturation of TCRs involved displaying a degenerate library of TCRs on the surface of M13 bacteriophage, followed by several rounds of increasingly stringent selection using immobilised cognate peptide-MHC complexes. This directed evolution approach, routinely applied to antibody production, could increase the TCR-peptide-MHC affinity by over a million-fold, through introduction of multiple mutations in the short hairpin loops, termed complementarity determining regions (CDR), that comprise the antigen binding site [14]. Yeast display, aided by computation modelling, has also been used to generate TCRs with enhanced binding affinity and/or stability [15–17].

Affinity maturation can enhance the natural affinity of anti-tumour TCRs (dissociation constant K_D range 10-100 μM [3]) by over a million-fold (K_D ~10 pM [14]). However, TCRs with affinities that are higher than the very strongest natural TCRs (K_D <0.1 μM) can activate T-cells irrespective of the cognate peptide [13,18,19] (**Figure 2A**). Additionally, engineered TCRs circumvent the rigours of thymic selection so that even slight alterations to TCR sequence may lead to unexpected cross-reactivities with antigens other than the cognate peptide-MHC. Such cross-reactivities can result in fatal adverse events [20–22]. Despite these caveats, TCRs with pM affinity towards cancer antigens could be used therapeutically as soluble molecules instead of in TCR gene-modified T-cells, without compromising the peptide specificity (**Figure 2B**). These super-high affinity TCRs can efficiently label cancer cells presenting endogenously processed peptide antigens (as few as 5 copies per cell [23]) and, when linked to an anti-CD3 antibody fragment, are capable of re-directing polyclonal populations of T-cells to kill cancer [24]. TCR-anti-CD3 antibody fusion proteins, termed ImmTAC™ (immune-mobilising monoclonal TCRs against cancer), have been developed by Immunocore (Abingdon, UK) and are being tested in six clinical trials, either as a single agent or in combination with checkpoint inhibitors (clinicaltrials.gov identifiers: NCT02889861, NCT01211262, NCT03070392, NCT01209676, NCT02570308, NCT02535078). A detailed description of how to manufacture super-high affinity TCR-anti-CD3 antibody fusion proteins has recently been published [25].

Bypassing thymic selection yields highly sensitive TCRs

Procurement of TCRs from T-cell populations that have not been selected against self antigens, such as HLA-transgenic murine models, provides a further way of generating high affinity cancer-specific receptors [11]. An extension of this methodology that avoids any potential immunogenicity of murine TCRs when transferred to human patients was proposed by Blankenstein and colleagues, whereby mice were humanised to express both a given HLA allele and the human *tcr* locus [26]. The lack of human antigens in these mice prevents deletion of high affinity human-reactive TCRs in the thymus. Thymic deletion can also be bypassed by using HLA-mismatched donors to generate allo-TCRs specific for a given peptide-MHC [27]. Allogenic TCRs are sensitive enough to target antigens expressed at a very low copy number that cannot be targeted by antibody-based therapies [28]. However, it is crucial to ensure that TCRs procured from allogeneic HLA donors are truly peptide-specific rather that recognising allo-HLA irrespective of the peptide cargo. Finally, enhanced affinity TCRs can be procured by antigen-driven differentiation of TCR-α transduced haematopoietic progenitor cells (HPCs) *in vitro*. Since HPCs undergo natural TCR-β rearrangement, the resulting TCRs do not contain any mutations in germline-encoded CDR loops, and non-germline encoded CDR3s can differ in length compared to parental TCR, making this approach potentially safer and more versatile than directed evolution of TCR proteins [29].

TCRs targeting tumour neoantigens are sensitive and specific – but at a price

Accumulation of somatic mutations is a hallmark feature of cancer. If non-synonymous mutations occur within protein fragments that can be displayed by MHC molecules, these potentially

immunogenic epitopes are termed “neoantigens” [30]. MHC-presented neoantigens offer an attractive target for immunotherapy as they are non-self, highly specific to cancer cells and circumvent thymic selection. T-cell responses against mutated antigens, but not the unmutated counterpart, can be sporadically detected in patients, and exploited to induce cancer regression [31]. Neoantigen-reactive T-cells can be identified non-invasively in peripheral blood of cancer patients using programmed death (PD)-1 expression as a biomarker prior to testing antigen specificity using mutations identified by high-throughput tumour exome and transcriptome sequencing [32]. Neoantigen-specific TCRs identified in this way can be subsequently used for personalised therapy. While targeting even a single point mutation with a specific TCR may be sufficient to eradicate established tumours, the particular mutation must be uniformly expressed by heterogeneous tumour cell populations and be essential for cancer cell fitness in order to avoid selection of escape variants [33]. Since cancer immunoediting ensures poor immunogenicity of potential driver mutations, at least at the early stages of cancer formation, endogenous T-cell pools may neglect the most therapeutically relevant mutations [34]. This problem can be circumvented by using HLA-matched healthy donors to procure TCRs against neoantigens that have escaped detection during the process of immunoediting [35].

Given the heterogenous composition of tumours and potential for immunoevasion when targeting only a single point mutation, it would seem optimal, although cost and labour-intensive, to target several neoantigens simultaneously, using an array of different TCRs. Emerging technologies may make targeting neoantigens more streamlined and applicable to larger cohorts of patients. Advances in high-throughput sequencing now enable rapid, non-invasive determination of mutations within the neoplastic exome [36]. Several cutting-edge methods have also been devised to infer the TCR specificity. We and others developed high-throughput methods for identification of cognate peptide ligands of TCRs of interest, using combinatorial peptide libraries [37,38] or yeast-display libraries of peptide-MHC [39]. Additionally, TCRs specific for predicted, patient-derived neoantigens can be identified and selected in a massively parallel way, by using DNA-barcoded peptide-MHC multimers, thus enabling screening of >1,000 putative peptide antigens in one sample [40]. This powerful technology can be further combined with single-cell transcriptomics [41], leading to rapid and streamlined identification of candidate TCRs. Finally, the most promising, neoantigen-specific TCRs can be introduced into patient-derived T-cells using a transposon/transposase system, offering a safer and cheaper alternative to currently used γ-retroviral/lentiviral vectors [42].

Perspectives – the brave new world of genome editing and synthetic biology

The ideal therapeutic T-cell should: (i) efficiently traffic to the tumour site; (ii) overcome the immunosuppressive microenvironment; (iii) successfully target all, potentially heterogeneous, tumour cells; (iv) be inert to healthy tissues; and, (v) be available as an off-the-shelf product that can be administered to patients regardless of their HLA allelic composition (**Figure 3**). While this may seem like wishful thinking, recent advances in synthetic biology and genome editing offer a very real potential to sculpt T-cells to fit a given therapeutic purpose. Tailoring T-cells to exact therapeutic needs can be achieved using artificial gene circuits, such as recently developed synthetic Notch receptors [43], engineered to release a selected transcription factor upon antigen binding to drive a bespoke transcription programme within the tumour [44]. T-cells can also be engineered to express two antigen receptors, with the second one becoming expressed only after productive antigen recognition by the first so as to limit the possible damage to healthy tissue [45]. However, the improved specificity of such dual recognition also amplifies the risk of cancer evasion. An off-the-shelf T-cell product would ideally be devoid of endogenous HLA to reduce the speed of rejection by the recipient immune system and express an antigen receptor that recognises cancer irrespective of

patient HLA type [46]. Removal of donor HLA and TCR is easily achievable by using programmable nucleases, and thus engineered allogenic T-cell products transduced with anti-CD19 CAR have recently been used in clinic [47]. Non-HLA restricted TCRs provide an alternative to CARs; in this respect tumour-specific γδ TCRs may prove more applicable than the conventional αβ TCR options that are currently being explored in the clinic. γδ TCRs can target a broad range of cancers but not healthy tissue - however, little is still known about possible ligands for these TCRs (reviewed in [48]). We have recently shown that using gene editing to remove endogenous αβ TCRs greatly enhances the anticancer activity of donor T-cells simultaneously transduced with γδ TCRs [49], and thus expect non-HLA restricted TCRs to be worthy of further clinical investigation.

Conflict of interest statement

Nothing declared.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

Acknowledgements

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Table 1 Completed clinical trials utilising TCR-transduced T-cells for cancer immunotherapy. Target refers to the protein from which the cognate peptide is derived (presented by HLA-A2, unless indicated otherwise) while the response rate indicates the number of patients experiencing objective clinical response out of all the evaluated patients. † denotes TCRs of murine origin; * presented by HLA-A1; ‡ presented by HLA-A24. MART, melanoma-associated antigen recognised by T-cells; NY-ESO, New York esophageal squamous cell carcinoma; CEA, carcinoembryonic antigen; MAGE, melanoma antigen gene.

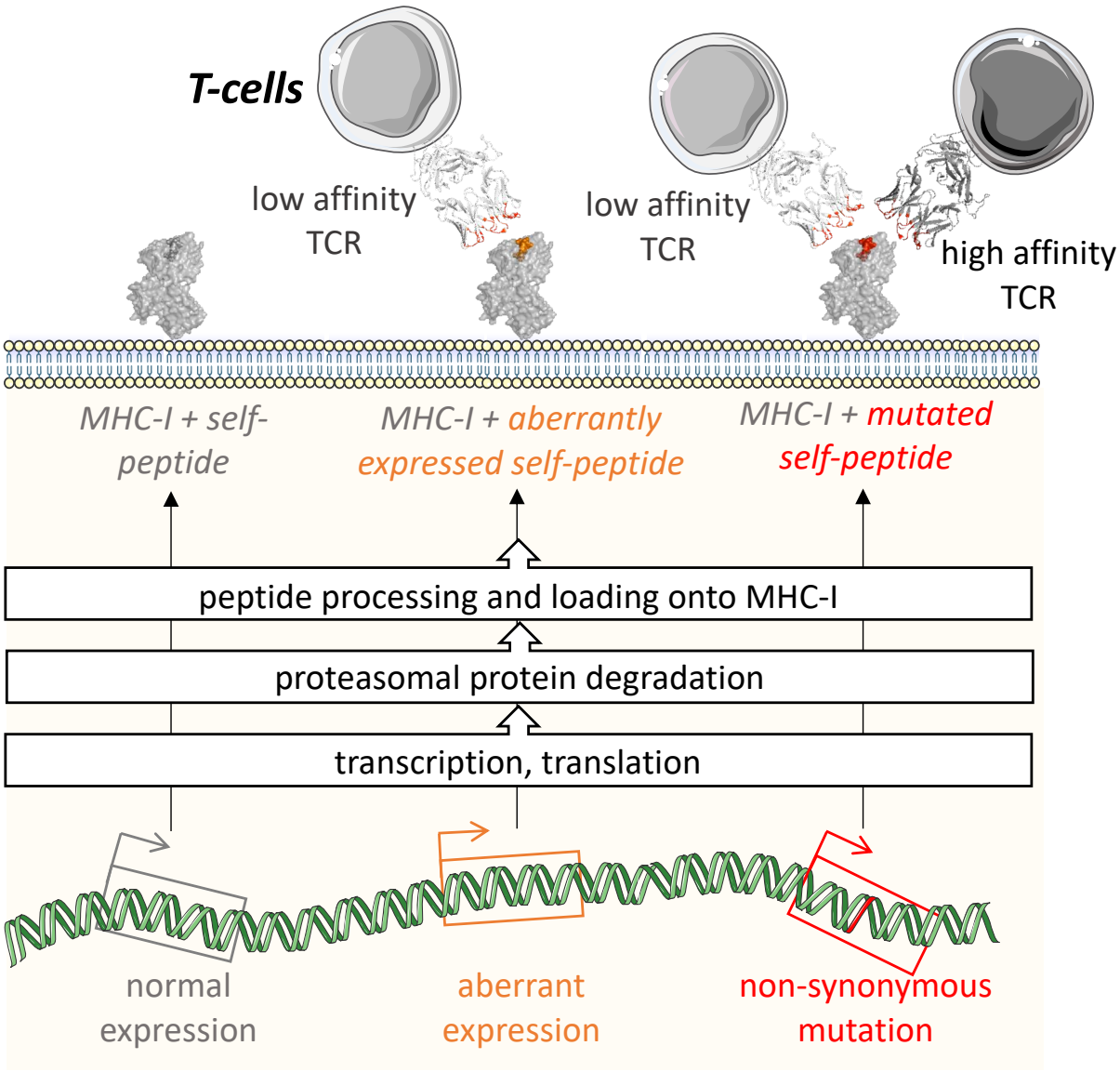
Target	TCR	Cancer	Response	Adverse effects	Reference
MART-1	Wild type	Metastatic melanoma	2/17	None	[10]
MART-1	Wild type	Metastatic melanoma	6/20	Grade 3	[11]
gp100	Wild type†	Metastatic melanoma	3/16	Grade 3	[11]
NY-ESO-1	Affinity enhanced	Metastatic melanoma, synovial cell carcinoma	9/17	None	[50]
NY-ESO-1	Affinity enhanced	Metastatic melanoma, synovial cell carcinoma	22/38	None	[51]
NY-ESO-1	Affinity enhanced	Multiple myeloma	18/20	Grade 3	[52]
CEA	Affinity enhanced†	Metastatic colorectal cancer	1/3	Grade 3	[53]
MAGE-A3	Affinity enhanced†	Metastatic melanoma/ synovial cell carcinoma/ oesophageal cancer	5/9	Fatality	[20]
MAGE-A3*	Affinity enhanced	Metastatic melanoma/ multiple myeloma	0/2	Fatality	[21]
MAGE-A4‡	Wild type†	oesophageal cancer	0/10	None	[54]

Figure legends:

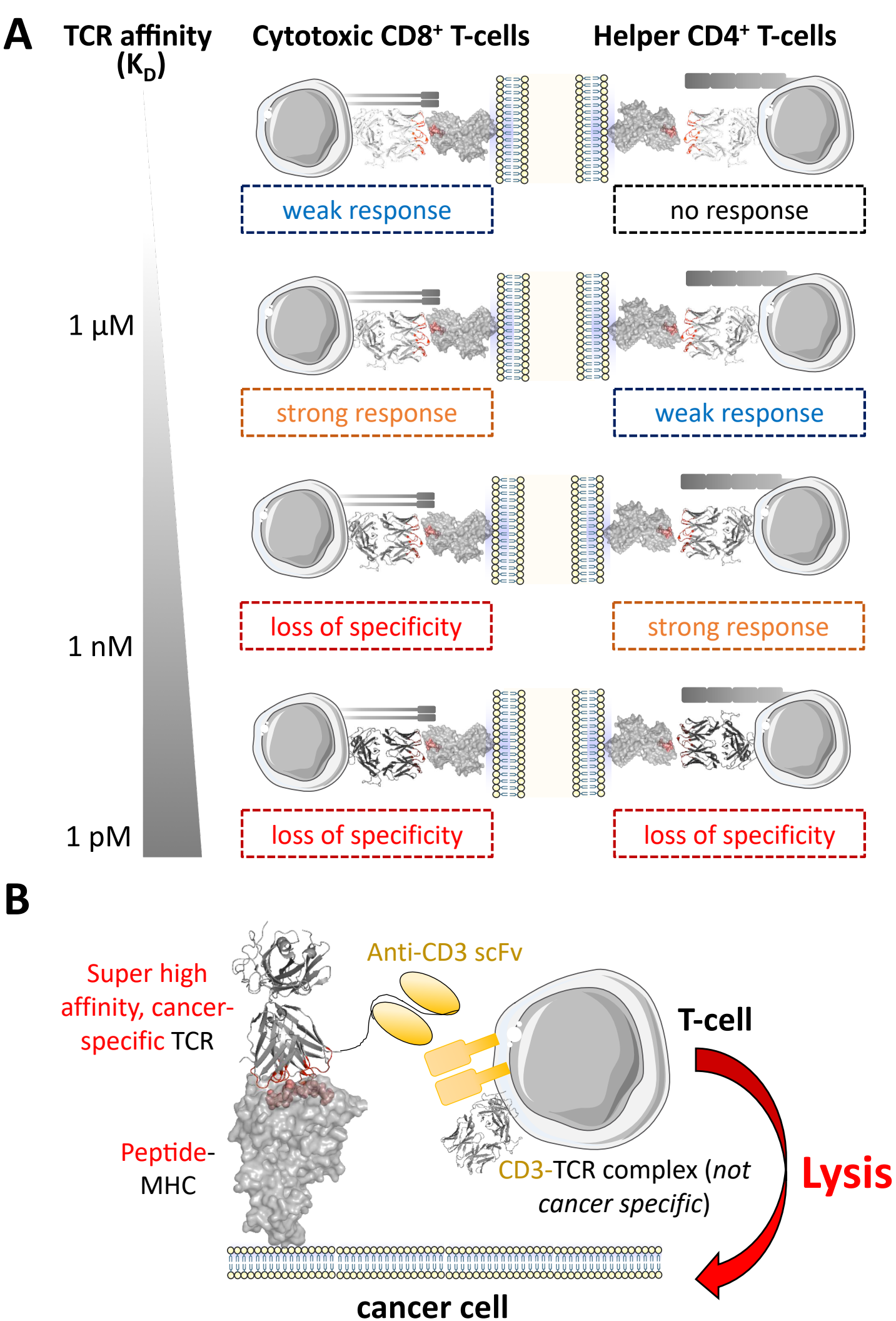
Figure 1. Natural anticancer responses consist of T-cells recognising short peptide antigens derived from aberrantly-expressed or mutated proteins. T-cells scan the intracellular proteome, presented as short peptides bound to MHC class I at the cell surface, using their hypervariable TCRs. The process of thymic selection prevents autoimmunity by culling T-cells bearing TCRs with high affinity towards peptides derived from self-proteins. Since many tumour-associated antigens are derived from self-proteins which are over- or aberrantly expressed (in particular, proteins expressed predominantly in immunologically privileged sites such as testes or developing foetus give rise to tumour antigens), thymic selection limits the pool of TCRs specific for these antigens to weak affinity, sub-optimal receptors. Thymic selection has little impact on TCRs specific for cancer-specific peptides arising from non-synonymous somatic mutations, as these are essentially foreign, highly personalised, antigens.

Figure 2. The affinity of TCRs towards cognate peptide-MHC can be enhanced – but requires tailoring to a particular application. (**A**) TCRs targeting cancer in context of peptide-MHC class I can be used for genetic re-direction of patient CD8 cytotoxic and CD4 helper T-cells. However, unmodified TCRs have a relatively weak affinity towards self/cancer-antigens – which may be sufficient to re-direct cytotoxic T-cells (which use CD8 co-receptor to enhance TCR binding to peptide-MHC-I) but not helper T-cells. As a result, CD4 T-cells require TCRs with higher affinity than CD8 T-cells to target cancer antigens via MHC-I without loss of specificity. Enhancing the TCR affinity to sub-nanomolar levels generally correlates with non-specific recognition of MHC molecules, when transduced into primary T-cells. Based on [17]. (**B**) TCRs with sub-nanomolar affinity towards their cognate antigens can be used as soluble molecules and are capable of binding to cancer cells with long half-lives. These super-high affinity TCRs can be covalently linked to single chain variable fragments (scFv) of an antibody (UCHT1) specific for CD3 molecule. Since CD3 is a component of the TCR, these bispecific reagents are capable of re-directing patient polyclonal T-cells to target cancer. Based on [23].

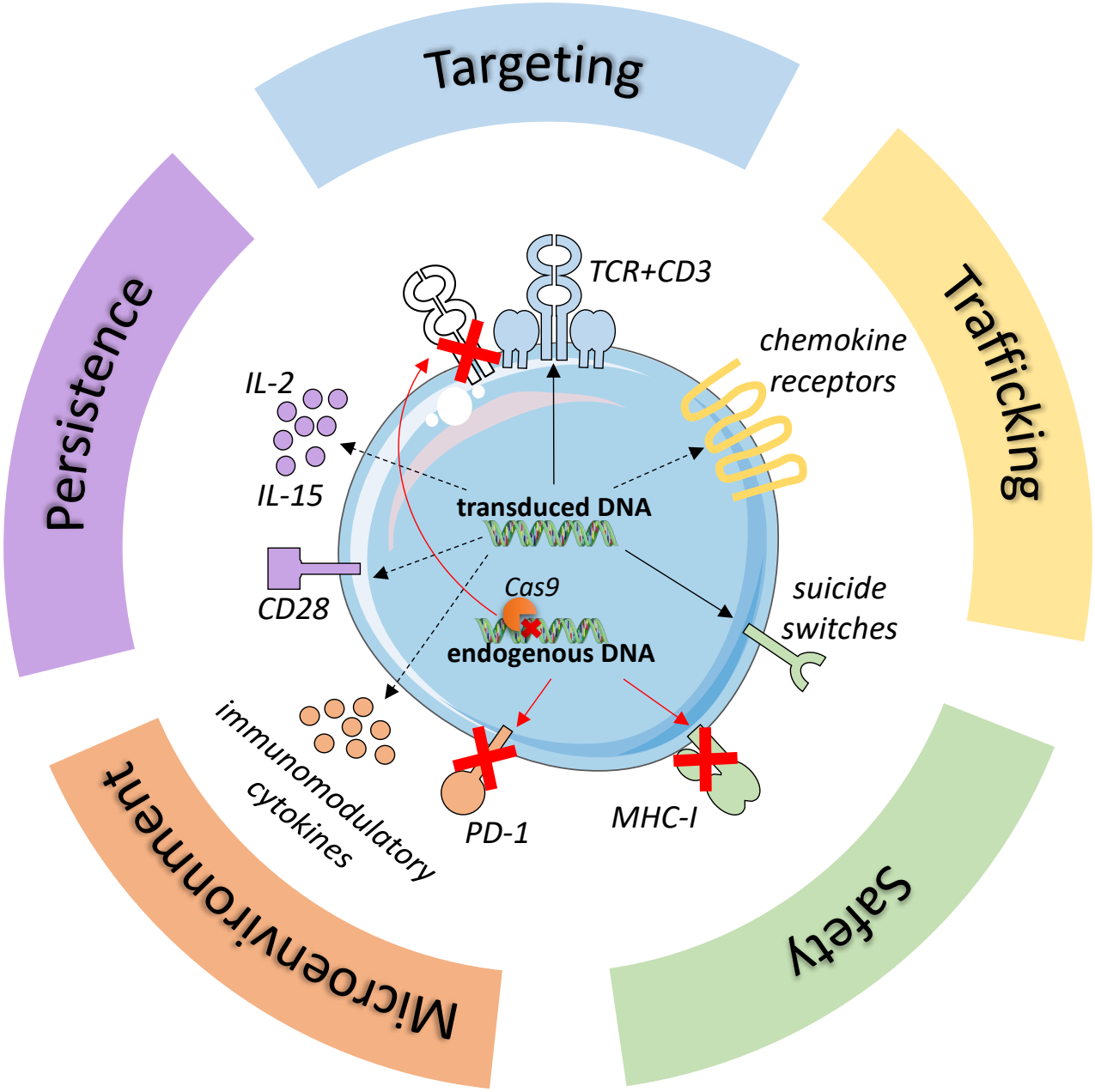
Figure 3. Hallmarks of an optimal cellular product for adoptive cell transfer. An optimal T-cell product can be generated by combination of gene knock-ins (for instance, delivered via a lentiviral vector) and knock-outs (transiently delivered in a form of Cas9 complexed with gRNAs). While Cas9 system can be easily multiplexed to target multiple genes at the same time, the amount of exogenous DNA that can be knocked in is limited by vector packaging constraints and cellular fitness considerations. Therefore, it may be advantageous to use an optimal T-cell subset for starting material, exhibiting the desired phenotype, cytokines and chemokine receptors. An alternative approach would involve using mutant Cas9 to activate endogenous gene expression. Finally, a safe off-the-shelf T-cell product should not express endogenous TCRs which can drive graft versus host disease, or endogenous MHC-I molecules which can lead to donor cell rejection. An optimal off-the-shelf T-cell product should target cancer, but not essential tissues, *via* a non-MHC restricted receptor to allow recognition in all patients.



Legut and Sewell, Figure 1



Legut and Sewell, Figure 2



Legut and Sewell, Figure 3